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PERSPECTIVES

Testing the theory of immune selection in cancers that break the rules of transplantation

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Abstract Modification of cancer cells likely to reduce their immunogenicity, including loss or down-regulation of MHC molecules, is now well documented and has become the main support for the concept of immune surveillance. The evidence that these modifications, in fact, result from selection by the immune system is less clear, since the possibility that they may result from reorganized metabolism associated with proliferation or from cell de-differentiation remains. Here, we (a) survey old and new transplantation experiments that test the possibility of selection and (b) survey how transmissible tumours of dogs and Tasmanian devils provide naturally evolved tests of immune surveillance.

Keywords Transmissible · Cancer · Immune selection · Epigenetic · Occult tumour · Tasmanian devils

Introduction

Immune evasion has been found in at least five animal cancer models. The panel includes (a) transplantation of methylcholanthrene (MCA) tumours from F1 donors to parental strain congenic mice [1, 2], (b) occult cancer

induced in mice by ultra-low doses of MCA [3], (c) immunoselection in metastases of transplantable mouse tumours, (d) the canine transmissible venereal tumour (CTVT) [4] and (e) the transmissible tumour of Tasmanian devils (devil facial tumour disease, DFTD) [5, 6]. Here, we survey these instances, enquiring how they bear at present on human cancer, and what they may reveal in the future. Our assumption is that intense selective pressure acting on these cancer cells evokes (via mutation and selection) a wide range of defensive strategies that enable them to survive immunological attack, as well as some that actively impair the host's attack machinery. The most important contribution from these animal models may be to validate the claims for immunoselection in cancer suggesting new avenues of research to investigate this problem in human cancers [7, 8]. Those modifications leading to immune escape that emerge in both the animal models and human cancers will be substantiated as likely due to immunoselection, while the status of those that do not do so will be called in question. The latter may to an unknown extent simply reflect de-differentiation or metabolic change associated with the proliferative activity of cancer cells. After all, changes in the level of MHC antigen (hereafter MHC) expression that in cancer cells are accepted as a hallmark of immune evasion also occur elsewhere, notably in foetal cells at the foetal–maternal interface [9], in embryonic stem cells and in neural progenitor cells [10–12].

Histocompatibility variants in mice: a historical perspective

During the 1920s, Little and Snell [13] began to explore systematically the use of inbred mouse strains to explore the rules of histocompatibility. They established that

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tumours could be successfully transplanted within an inbred strain and its F1 hybrids, but not into other strains. Tumours originating in an F1 hybrid could be transplanted within the same hybrids but not into either of the parental strains. Thus, Haldane noted, the histocompatibility factors that governed transplantation behave as primary gene products that might serve as antigens, a suggestion that was later verified by Gorer and Snell. Snell [13] continued the analysis of histocompatibility by breeding mouse strains that differed from one another at single histocompatibility loci, his so-called congenic strains (although we now know that each MHC is a composite of several closely linked genes). The availability of these congenic strains prompted two groups to use them to test the genetic stability of these MHC antigens [1, 2]. The studies were carried out in different mouse strains and were entirely independent, but yielded essentially identical results illustrated in Table 2. Prior to rejection in an MHC incompatible strain tumour transplants grow for a few days, thus generating a population of cells that come under intense selection for loss of their MHC antigens. Both studies found that under these conditions, tumours of F1 origin (i.e. MHC-heterozygous) regularly lost expression of the MHC antigen(s) foreign to the host, whereas MHC-homozygous cells failed to do so. The loss was permanent and heritable, remaining evident after passage through the neutral F1 host. It contrasts with the consistent behaviour of the many MHC-homozygous tumours that Snell had used to derive the MHC-congenic strains, where antigen-loss would have been fatal to his enterprise. Loss of the MHC antigens was further validated by serology, showing that the variant tumour cells neither reacted with antisera directed against the missing MHC antigens nor proved to be able to elicit such antibodies [2]. Furthermore, the variant cells grew in pre-immunized hosts, where even weak expression of the missing antigens would have been detected. Providing further evidence of mutation, the cells derived from heterozygous tumours increased their frequency of take in the parental hosts after X-irradiation. Thus, the evidence for genetic change (or possible stable epigenetic change) is strong, but in neither study was this the only possibility. Not all F1 tumours yielded straightforward allele-loss variants [2], as found in the small study illustrated in Table 2 taken from [1].

These findings were interpreted at the time in terms of single gene mutation. Later, this interpretation was excluded when the mouse MHC was found to include several major polymorphic genes (H2K, H2D, H2A, H2E). It now seems likely that the loss of heterozygosity (LoH) occurred through recombination, although chromosome loss may also have contributed, as previously recorded in long-transplanted “non-specific” mouse tumours by Sachs and Gallily and by Hauschka and Levan, in early work cited in [2]. These early studies are precursors of the systematic

work on human tumours that has revealed that both chromosome loss and somatic recombination occur frequently in human tumours [14]. A recent development is the molecular characterization of a recombination hot spot in the mouse MHC, which is present in some but not all haplotypes [15].

Occult sarcoma induced by low dose methylcholanthrene

Treatment of mice with ultra-low doses of the carcinogen 3'-methylcholanthrene (MCA) induces not only a few cancers of usual type, but also some of occult type that grow out only in the presence of concomitant immunosuppression [3]. This does not occur if the mice are immunocompromised right from the start of tumour induction, using RAG-knockout mice. In further tests, certain monoclonal antibodies proved effective in compromising this selection mediated by the immune system (as annotated in Table 1), while others did not. The system represents an extension of a broad range of previous work demonstrating that MCA tumours in mice undergo immunoselection, cited in [3]. It is likely to prove useful in future work, for sorting out the modalities of immune intervention able to mediate or inhibit selection of tumour variants. We look forward to a comprehensive annotation of Table 1, noting validation or exclusion over an expanded range of evasion mechanisms.

Metastases of chemically induced mouse tumour undergo immunoselection in normal but not in athymic (nude) mice

Metastatic tumour variants derived from transplants into normal hosts regularly lost MHC class I expression, while cells from similar transplants into immunocompromised (athymic nude) mice did not do so [16]. The loss of MHC expression is associated with loss of mRNA for the APM proteins identified in Table 1.

Canine transmissible venereal tumour (CTVT) and transmissible tumour of Tasmanian devils (DFTD)

CTVT was first described in 1876 by Novinski [17] and is a transmissible cancer. Natural transmission of CTVT between dogs usually occurs through coitus, but also by biting or licking tumour-affected areas [18]. The tumour can also be transplanted experimentally between dogs. Generally, CTVT grows on the external genitalia and may also metastasize internally and spread to other mucosal surfaces, although this is a rare event, occurring mainly in puppies, immunodepressed or previously sick dogs [18].

Table 1 Candidate mechanisms of immune evasion

Gene	Function
MHC I (HLA-A, B, C) ^d [44, 67, 68]	Targeting of CD8 T cell (CTL)
β_2 -Microglobulin ^d [44, 69]	MHC I expression
Proteasome components: delta, MB1, Z; LMP's ^c [45, 69]	Antigen processing machinery (APM)
Chaperones: calnexin ^c , ERp57, calreticulin ^d [69]	
Peptide transporters: TAP1 ^c and TAP2 ^c [45, 69]	
(MHCII) HLA-DR [43]	Targeting of CD4 T cell
Whole MHC (Loss of heterozygosity) [27, 28]	Immune evasion
HLA-G [70]	Targeting of regulatory T cell (Treg). Inhibits NK-cell function. Protects trophoblast
Deaf1 transcription regulator [46]	Suppresses expression of peripheral tissue antigens
FoxP3 ⁺ Tregs [51, 71]	Protect against autoimmunity
CTLA-4 [72, 73]	Down-regulatory
Th17 cells [74]	Modulate other tumour-infiltrating T cells
KIRs and other NK-cell receptors ^b [47, 75]	Modulate NK cells
NKG2D ^b and other NKT-cell receptors [48, 76]	Activating receptor on NK and NKT cells
IL12 [77] ^a	Required for NKT-cell activation
TGF β 1 [78]	Immuno-regulatory
IL-10 [77]	Immuno-regulatory
IDO indoleamine-2,3-dioxygenase [79, 80]	Immuno-regulatory
IFN- γ [79]	CTL-upregulatory
IFNGR down-regulation, truncated dominant- negative form [81]	Down-regulatory
Fas Ligand [82]	Mediates Fas–FasL CTL cytotoxicity
TRAIL [82] ^b	Mediates CTL cytotoxicity
STAT3/STAT4 [83]	Promotes tumour growth, inhibits immunity

^a Effective target in the occult cancer system [3]

^b Ineffective in the occult cancer system

^c Effective target in the mouse metastasis system [16]

^d Ineffective in the metastasis system [81]

Recent genetic evidence conclusively showed that the tumour cell itself is the transmissible agent responsible for CTVT [4] and, on the basis of microsatellite and mtDNA variation analysis, it has been possible to estimate the age of CTVT at between 250 and 2,500 years or earlier [4, 19]. CTVT thus represents the oldest known cancer cell lineage. Genetic analysis on microsatellite and DLA alleles indicate that CTVT most likely originated in wolves and it has been hypothesized that the inbred nature of some wolf groups might have facilitated the initial spread of this transmissible cancer, similar to the facial tumour in Tasmanian devils [4]. However, further evolution towards immunological escape must have occurred in CTVT to allow its spread to a wider dog population.

Remarkably, transplanted CTVT shows a phase of progressive growth, followed by spontaneous regression after 3–9 months in most dogs, unless the animal is in poor condition [18, 20]. Hence, CTVT is characterized clinically by a progressive, a stationary and regressive phases. Recovered dogs are immune to tumour growth upon

reinoculation. Irradiation of dogs before CTVT is transplanted experimentally increases tumour malignancy, presumably due to host immunosuppression [20]. The pathology suggests that tumour-infiltrating lymphocytes (TIL) and macrophages may promote CTVT regression [21, 22] and overall, the available data suggest that the immune system plays an important role in regression of CTVT. Because CTVT is clonal and it is genetically stable [4], we hypothesize that changes leading to regression must be regulated epigenetically.

The DFTD is another form of transmissible cancer that is becoming highly prevalent in Tasmanian devils (*Sarcophilus harrisii*), a marsupial carnivore widespread in Tasmania [23]. First described in 1996, DFTD appears to be more recent than CTVT and, in contrast to CTVT, does not regress. In fact, DFTD is almost invariably deadly within 6 months of transmission and is causing a rapid decline of the devil's population [23]. The tumour affects facial areas and causes death presumably by suffocation and extreme difficulty in feeding. Transmission occurs by

biting in the facial area during frequent fights or mating. Genetic and karyotype analyses indicate that DFTD is also transmitted as a cellular parasite, though the evidence supporting its clonality is not as strong as in the case of CTVT [24, 25]. DFTD transmission within the devil population is presumably greatly facilitated by the low diversity of MHC alleles [25]. Whether DFTD is subject to immunoselection has been questioned, on the grounds that the population of devils in which it occurs lacks diversity at the MHC as a consequence of demographic factors: a population bottleneck and inbreeding. Wild devils show limited sequence diversity at the MHC, and their mixed lymphocyte reaction (MLR) is low [6]. However the crucial test of skin graft survival has not been applied, so the spread of DFTD may yet reflect, in part at least, immunoselection of the tumour phenotype.

Mechanisms of immune evasion

The main genetic mechanisms thought to mediate immune evasion are listed in Table 1. Although not comprehensive, we suggest that this list may help guide future research on immunoselection in the CTVT and DFTD models presented here. It is likely that future work in these models will rely largely on molecular and DNA technologies, so we mention that apart from HLA-G, all the genes listed in the table have known orthologs in the dog, and nearly all also in the opossum, the closest relative of the Tasmanian devil included in the ENSEMBL database. In this connection, we return to the caveat already mentioned. The loss of a key immunological molecule such as an MHC is not necessarily a consequence of immunoselection, since cancer cells may simply refocus their metabolic machinery elsewhere. Cancer cells may also undergo a progressive de-differentiation process, becoming more similar to embryonic stem cells or to some stem cell types that express very little if any MHC class I and II molecules on their surface [10–12]. The other caveat is that CTVT and DFTD are allografts, hence they may be subject to greater immunological selective pressure than normal cancers; yet these transmissible cancers have been in continuous propagation in natural conditions over long period of time and are therefore likely to be very informative on the evolutionary strategies developed by cancer cells to evade immunosurveillance.

Loss of MHC genes

The high level of variation at and around the MHC makes LoH (loss of heterozygosity) relatively easy to detect. Frequent loss has been detected in biopsies [14] and in cell lines from carcinoma [26] and melanoma [27, 28],

and in leukemic blasts from relapse but not from fresh cases [29]. A recent study [30] provides a spectacular example of LoH in man, where cancer cells passed across the placenta lose the entire MHC haplotype not inherited by the infant.

In comparison, little is known about LoH in normal cells. An obvious question is whether it occurs when HLA-heterozygous bone marrow cells after haploidentical bone marrow transplantation, now a common form of therapy in cancer and (more rarely) in congenital hemopoietic disease [31]. LoH should be detectable by FACS analysis [29], provided that the transplanted cells can be identified by a marker other than their MHC. An extensive recent study of transplantation of haploidentical donor T cells for acute myeloid leukemia or myelodysplastic syndrome provides a striking example of immunoselection. Relapse was associated with the presence of mutant variants of the original leukemic cells, in which the HLA haplotype that differed from the donor's haplotype had been lost [32].

LoH could not account for the ability of CTVT to grow in a wide range of hosts, although it could enable it to grow in a limited range of closely inbred dog breeds. In principle, LoH might have contributed to the initial transmission of the tumour within members of closely inbred wolf groups [4, 19]. Genetic analyses showed that CTVT has the same DLA haplotype worldwide and that most class II DLA genes were diploid in CTVT, except the DQB1 and DQA1 loci, which were haploid but only in about 50% of the tumours analysed [4]. Thus, even if LoH is relatively frequent in CTVT, it does not seem to have contributed to a founder effect and we suppose that this would not provide a significant selective advantage, at least in this model.

Reduced MHC class I expression

MHC class I (MHC I) expression can be suppressed by loss of expression of β_2 -microglobulin (β_2 M). β_2 M is an invariant single domain protein that binds to and stabilizes the larger 3-domain, antigen-binding, variable α -chain component of MHC I. Loss of β_2 M causes loss of MHC I expression. Attention is now directed also to the antigen processing machinery (APM), where the key molecules responsible for reduced expression in tumours are listed in Table 1. As a rule the proteasome itself cannot be modified, because it forms a key part of the machinery for eliminating defective self-proteins, but the self-peptides produced by proteasomes, prior to binding to MHC I molecules pass down a chain of auxiliary molecules that are often down-regulated in cancer cells.

CTVT has been shown to have little β_2 -microglobulin expression, at least in the progressive phase and very low MHC I expression [33, 34]. Whether or not the APM is also affected is not known. Tumour cells in the so-called

regressive phase re-express normal levels of MHC I and β_2 -microglobulin [34, 35]. This suggests that loss of both MHC I and β_2 -microglobulin expression is important for CTVT transmission and that its regulation is likely to have an epigenetic mechanism. Remarkably, MHC I suppression in CTVT is not complete and, in fact, is modulated presumably to prevent recognition and killing by NK (natural killer) cells [4]. This “fine tuning” may be the result of the powerful selective bottleneck during transmission of CTVT, whereby any transmitted cell lacking MHC I would be rapidly eliminated by NK cells. Hence, selection might have occurred against deletion or deleterious mutations in MHC I genes in the case of CTVT. Whether these kinds of positive and negative selective pressures on the MHC I genes are also exerted in other cancer cells is unclear at present. If widespread, such a finely balanced regulation of MHC I expression would provide strong evidence for a primary role of the immune system in cancer evolution.

Reduced MHCII expression

MHCII expression is more variable than expression of MHC I, both within an individual immune system and at the population level, and most cancers and normal tissues do not express MHCII. MHCII is essentially a receptor that enables lymphocytes to communicate with one another, and is therefore expressed only on a limited range of cells. In contrast, MHC I allows cytotoxic T cells (CTL) to target any cell infected with a virus or other intracellular parasite, and therefore has ubiquitous expression. Nevertheless, CD4⁺ MHCII-restricted T cells engage in anti-tumour responses [36], and offer possibilities for therapy [37]. CTVT expresses little MHCII antigens on the cell surface in the progressive phase, although this phenotype is reversed in the regressive phase [21]. Interestingly, dogs that have overcome CTVT are immune to re-inoculation and have antibodies that recognize antigens on the surface of CTVT cells, which may drive acute rejection [35, 38]. Expression of MHC class II antigens by CTVT cells is likely to promote the generation of CTVT-specific antibodies, suggesting that re-expression of MHCII in the regressive phase may have an important role in inducing subsequent protection against re-inoculation. Similar to MHC I antigens, the regulation of MHCII expression in CTVT is likely to be epigenetic in nature but in this case it is unclear why MHC II genes have not undergone deletion during the evolution of this cancer.

Much of our understanding of MHCII gene expression derives from the bare lymphocyte syndrome (BLS), a rare MHCII deficiency in which CD4⁺ T cells fail to develop, thus increasing susceptibility to infection [39]. The mutated genes encode three regulatory (RFX) factors that bind to

DNA motifs upstream from the genes encoding the MHC molecules, and one non-DNA binding protein (CIITA) that binds the RFX factors together into a regulatory complex. Thus, the CIITA protein functions as a master switch controlling MHCII expression, which also regulates MHC I expression. These factors themselves are not polymorphic, but their binding sites in the promoter region upstream of the structural genes have high levels of natural variation, comparable to that of the MHC structural genes themselves [40, 41]. Thus, these regions upstream of MHCII genes in the venereal tumours deserve attention. On the other hand, the Tasmanian devil’s facial tumour cells appear to express both MHC class I and II genes, and transmission of this tumour may be better explained by the lack of MHC diversity in the devil population. It should, however, be noted that MHC expression in the tumour cells was examined only at the mRNA level and one cannot exclude defects in protein expression or localization at the cell surface. If, however, lack of MHC diversity in the devil’s population really explains transmission of the tumour, DFTD can be used as a negative control to support the concept of cancer immunoselection: DFTD does not repress MHC genes because it may not need to.

Epigenetic control of MHC expression

All of the MHC genes mentioned in Table 1 are doubtless subject to the standard machinery of epigenetic control, including DNA methylation at CpG islands and histone methylation, phosphorylation and ubiquitination [42–44]. In addition, the role of histone deacetylases (HDACs) in down-regulation of MHCII gene expression has been well studied and occurs through at least two major pathways [45]. Recruitment of HDAC1 and HDAC2 causing disassociation of CIITA from the MHCII gene promoter and recruitment of HDAC4 via interaction with RFX-associated ankyrin-containing protein (RFX-ANK) both cause MHCII silencing. Inhibitors of HDAC such as trichostatin-A enhance MHC expression, and thus provide a means for exploring epigenetic MHC-silencing [45].

Dendritic cells

Among the many possible modes of interventions in antigen presentation, events at dendritic cells are of special interest because of the ambiguous capacity of these cells, in activating T cells while at the same time being able to recruit other down-regulatory T cells, such as Tregs [36]. New work on the autoimmune disease TD1 (type 1 diabetes) reveals Deaf1 to be a key regulator of antigen presentation in these cells [46]. Although not yet studied in cancer, this molecule would seem to be a promising

candidate for inclusion in Table 1. Little is known of dendritic cells in CTVT or DFTD.

NK cells and NKT cells

Natural killer [47] and NKT [48] cells (distinct populations) attack certain tumour cells without prior activation, hence their name. They express a wide range of stimulatory and inhibitory receptors, including the KIRs. Some KIRs are MHC-restricted, and so act more effectively when tumour cells have reduced MHC expression. Other NK receptors do not interact with the MHC but may nevertheless mediate immunoselection. The NKG2D (HLA-G-binding) receptor is a well-characterized non-polymorphic down-regulatory receptor. HLA-G is a non-variable HLA-I-type protein, found cell surface-bound and in body fluids, and thought to mediate tumour immune evasion [49]. As mentioned above, NK-cell activity was not evident with the occult tumours (annotation in Table 1). The role of NK cells in CTVT regression is not fully understood but there is evidence suggesting that their activation is important, at least in tumours transplanted in SCID mice [50]. Recruitment of NK cells with CTVT killing activity appears to require high levels of both IL-6 and IL-15 [50].

T-regulatory cells

The role of CD4⁺CD25⁺FoxP3⁺ Treg cells in transplantation is a subject of much current interest [51]. These cells comprise two forms, one generated in the thymus and naturally occurring and the other adaptive. Their mode of action may well be competitive uptake of cytokine(s) needed for proliferation [52]. Thus, mopping up of the cytokines needed for a positive response would make sense as a protective mechanism in the transmissible tumours. Other T cells with immunosuppressive activity include CD4⁺ IL-10 secreting cells, CD4⁺ TGF- β secreting cells and Th17 cells. There is evidence that CTVT cells themselves secrete substantial amounts of the down-regulatory cytokine TGF- β (see Table 1), which is perhaps expected of a tumour that shows evidence of histiocytic origin [53, 54]. A high local concentration of TGF- β has been shown to protect tumour cells from infiltrating CTLs, possibly by suppressing their activity and by contributing to the tumour's MHC I and II down-regulation [55]. Progressive and regressive CTVT cells secrete similar quantity of TGF- β 1, yet they show dramatic differences in MHC expression [21, 35, 55]. Moreover, CTVT cells seem to be unresponsive to IFN- γ produced by CTLs [55], which may, in part, explain their low MHC I expression levels in the P phase [56]. It has been proposed that higher levels of IL-6 secreted by infiltrating CTLs could counteract the immunosuppressive activity of TGF- β 1 and kick start the

regressive phase [57]. However, it is unclear why infiltrating CTLs should express higher levels of IL-6 at a certain time during the clinical evolution of CTVT.

The observation that TGF- β 1, and perhaps other immunomodulatory cytokines, is secreted by CTVT cells [55] might explain why the venereal tumour rarely metastasizes to internal organs, even though it is perfectly able to do so in immunocompromised dogs. It is likely that a threshold of TGF- β 1 concentration needs to be reached within the tumour microenvironment to be protective and that tumour cells circulating in the blood stream are unlikely to reach such levels.

Discussion

In summary, lessons from the panel of animal model systems suggest that multiple elements contribute to the lack of cancer immune rejection. They include repression of MHC class I and perhaps MHCII antigens also, at least in the case of CTVT [34, 35], concomitant loss of β 2-microglobulin and secretion of immunomodulatory cytokines that promote tolerance. Additional mechanisms, such as polymorphisms in MHC gene promoters, NK-cell and NKT-cell activities and modulation of CD4⁺ Treg cells, though more speculative, deserve further investigation. Whether all these mechanisms are solely consequence of immune selection is not completely clear for some of them (MHC down-regulation), and may be a consequence of loss of differentiation typical of cancer cells. Interestingly, re-expression of MHC I and II molecules in regressive CTVT coincides with an apparent differentiation of tumour cells [35]. Hence, de-differentiation of cancer cells towards an embryonic stem cell-like type may favour immunoevasion and immune surveillance may, in turn, favour the evolution of such an undifferentiated state of cancer cells.

The evidence for immunoselection is least clear for DFTD, where the alternative hypothesis of loss-of-diversity in the host is tenable. The special value of having five

Table 2 Transplantation of a newly induced methylchloranthrene sarcoma

B10	F1	B10.D2	F2
3/8	Original F1 line 10/10	2*/8	9/18
B10 sub-line 7/7	3/3	1/4	
5/9	3/3	B10.D2 sub-line 3/4	

Induced in B10 \times B10.D2F1 mice, and its sublines, derived from takes in the parental strains. Proportions of takes shown are tumours grown to 1 mL at the time of killing, within 4 weeks of transplantation [with one exception (asterisk), where the transplant took 8 weeks to grow]

such systems now available is that immunoselection occurs over such different lengths of time. The F1 tumour transplanted to parent usually develops within 4 weeks, prior to killing (Table 2). The microdose-MCA occult tumours become evident in mice after 200 days, although the timing of their emergence is not known in detail. The metastatic tumours develop over a few weeks. In contrast, both CTVT and DFTD are ancient, with long exposure to immunoselection in serial hosts. These various systems are thus likely to reveal mechanisms of evasion that differ significantly but may well overlap. It is of particular value to have CTVT included, because as a “histiocytic” tumour it is potentially able to express MHC class II and therefore reveal immunoselection acting on these molecules (Table 1).

Future work will no doubt further examine the occult and metastatic MCA tumours, beyond the annotation of effective and ineffective treatments illustrated in Table 1. Immunosuppressive drugs should prove informative here. In the search for inhibitors of organ transplant rejection, drugs with diverse modes of action have been identified [3, 58, 59]. These include (a) inhibitors of T cell activation, such as calcineurin inhibitors, LFA-1 inhibitors and inhibitors of CD28 binding to CD80/86, (b) T cell depleters, such as inhibitors of T cell proliferation, inhibitors of nucleotide or purine synthesis and inhibitors of mTor and (c) blockade of IL-2 binding to IL2 receptor. Any of these agents might interfere with immunoselection and thus hinder tumours with reduced antigenicity from emerging.

These are certainly not the only strategies of interest for probing the latent state of the occult tumours. It would be of interest, for instance, to apply anti-cancer drugs that target cell proliferation. These might rid the mouse of its non-occult tumour cells, and thus allow the occult ones to be studied at early time points in the course of their selection. A further possibility would be to apply chemical mutagens such as ENU (*N*-ethyl-*N*-nitrosourea) to evaluate the role of gene mutation in the response to immunoselection.

Another future topic is TIL. Recent surveys of TILs in head and neck cancer [60], ovarian and colon cancer [61, 62] report that systematic analysis of CD4⁺ and CD8⁺ T cell infiltrates has significant prognostic value. More generally, a multiparameter complex of readouts including histology, gene expression profiling and cytokine profiles is providing improved survival predictions [63]. TILs have not yet been found in DFTD, presumably because of the low level of immunity [6] but regressive CTVT is infiltrated by TILs, supporting a role for these cells in cancer immunosurveillance [64].

Importantly, the CTVT model shows that, in certain circumstances, some of the elements important to evade immune-recognition can be reversed and are therefore likely to contribute normally to tumour regression. Do any

or all of these various evasion strategies depend on a single “master switch”, and is such a switch open to appropriate intervention? These are central questions in both organ transplantation and cancer. In CTVT, concomitant down-regulation of MHC I and II with secretion of immunomodulatory cytokines suggests two alternative hypotheses. Either MHC down-regulation is insufficient on its own to permit immune evasion and needs an additional layer of protection, or the secreted cytokines directly or indirectly control MHC expression. The first scenario would provide a selective advantage if CTVT cells exist in a quasi-stable epigenetic state, where a proportion of them re-express MHC molecules at any one time. In that case, secretion of immunomodulatory cytokines would provide a second line of protection against immune rejection by promoting tolerance [65]. The second scenario more easily explains why MHC down-regulation is reversible in CTVT. It does not, however, easily explain why CTVT cannot generally escape immunorejection once the process has started. We speculate that only a subset of CTVT cells makes enough immunomodulatory cytokines to prevent rejection, and once these cells are gone the tumour enters an irreversible regressive phase. The role of cancer cell differentiation in the regression of CTVT and its impact on immune-recognition is also worth investigating. Cancer stem cells are often defined as a population subset able to grow tumours when serially transplanted in immunocompromised mice [66]. However, it has become apparent that the degree of immunocompetence of the recipient mice plays a role in establishing the frequency of cancer stem cells in a given tumour [66]. Little is known on the role, if any, of cancer stem cells in promoting tumour immunoevasion. Perhaps, similar to cytokines-secreting cells in CTVT, cancer stem cells might contribute to evasion in this way. On the other hand, the immune system may play a role in selecting in the early phases of tumourigenesis some cancer cells with undifferentiated and stem cell-like properties. Although little is known on the (reversible) mechanisms underlying MHC class I antigen down-regulation in embryonic stem cells, some of these mechanisms may turn out to be the same in CTVT and more broadly in other types of cancer cells. In this respect, CTVT, which is a natural experiment of cancer cells transplantation, may also provide a valuable model to investigate the relationship between cancer stem cells and immune surveillance.

Some of these important questions can now be addressed by high throughput analyses to compare progressive and regressive CTVT samples and look for genes differentially regulated in the two phases. Coupled with genetic sequencing of the entire CTVT genome and detailed immunohistopathological analyses, this approach is likely to reveal important gene candidates responsible for CTVT regression and will allow extension of such analyses to human cancers.

The available evidence in CTVT suggests that epigenetic regulation of crucial immunological genes such as MHC I, MHCII and β_2 -microglobulin may offer an evolutionary advantage to cancer cells superior to deletion or mutation of these genes. If this possibility is verified and applies in other cancers, it would open the way to novel therapeutic options aimed at subverting the epigenetic structure of cancer cells.

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